Full Title:

Pilot study to evaluate the effect of a calcium and vitamin D-containing oral bolus on serum calcium levels in early lactation Holstein dairy cows

Short title:

Calcium supplementation and serum calcium in early lactation dairy cows

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Abstract

The initiation of lactation challenges the ability of the modern lactating cow to maintain calcium homeostasis, and typically results in a drop in blood calcium levels; leading to mobilization of calcium reserves from skeletal stores. As such, the recommendation to provide supplemental calcium at parturition to older cows has become an industry-standard practice. Mature cows were treated at calving and 12 hours later with either the novel calcium bolus (Calboost (CB) (Solvet, Calgary, Alberta, Canada)), or a commercially available calcium bolus (Bovikalc (BK) (Boehringer Ingelheim Vetmedica Inc., St. Joseph, MO)). Blood was collected from animals at 0, 1, 6, 12, 13, and 24 hours following calving, and the resulting serum samples were analyzed. Overall, there was no statistical difference between the two calcium boluses for blood calcium levels within the first 24 hours following parturition (P = 0.50). Cows treated with both oral calcium boluses experienced a significant increase in serum calcium by 1 hour after parturition, however, this increase was not sustained through subsequent sampling times. This pilot study demonstrates that both boluses have a similar effect in the elevation of blood calcium.

Introduction

The initiation of milk production challenges the ability of the lactating dairy cow to maintain normal calcium concentrations. Once lactation begins, blood calcium levels drop leading to mobilization of calcium reserves from skeletal stores [1,2]. Clinical and subclinical hypocalcaemia are common issues in the dairy industry [3–5], and are both associated with a variety of negative sequelae including an increased risk of displaced abomasum, mastitis, retained placenta, dystocia, and ketosis [6,7]. Given these negative connotations, it is imperative to improve the calcium status of a newly freshened dairy cow.

50 Oral supplementation with calcium chloride and calcium sulfate in a fat coated bolus has shown to have
51 significant effects on improving calcium status in the period following calving [8]. Clinically, Oetzel and
52 Miller [9] found that the administration of the bolus to lame cows and high producing cows lead to
53 fewer health events and higher milk production in these groups, respectively leading to an economic
54 benefit of $3 to $8 per cow [10]. There has been very strong adoption of the oral calcium supplement by
55 dairy producers throughout North America; however, many of the alternative products to Bovikalc
56 (Boehringer Ingelheim Vetmedica Inc., St. Joseph, MO) have not been tested to demonstrate similar
57 efficacy in influencing blood calcium levels.
58 Therefore, the primary objective of this pilot study was to assess the ability of a novel oral calcium bolus
59 containing calcium chloride, calcium sulfate, and vitamin D3 (Calboost [Solvet, Calgary, Alberta, Canada])
60 to influence the serum calcium level in recently fresh, mature Holsteins over the first 24 hours following
61 parturition, relative to a positive control (Bovikalc). We hypothesized that there would be no difference
62 between the two oral boluses with respect to serum calcium levels over the first 24 hours following
63 administration.

64 Materials and methods

65 Ethics statement

66 The present field-based study was conducted in compliance with the research guidelines set forth by the
67 Canadian Council on Animal Care after appropriate Animal Use Protocol review by the Institutional
68 Animal Care and Use Committee (Alberta Agriculture, Airdrie Alberta, Canada).

69 Sample size calculation

70 Based on previous research, oral calcium supplementation should increase the serum calcium
71 concentration by 0.25 mmol/L (0.125 mmol/L standard deviation) relative to levels at calving [11]. A

72 total sample size of 20 mature cows (10 each in Calboost and Bovikalc groups) was calculated, assuming
73 a 95% confidence level and 80% power.

74 Study herd and treatment protocols

75 The study was conducted at a commercial dairy farm milking 500 cows in southwestern Ontario,
76 Canada, between March and September 2018. Average milk production per cow was 38 kg, at 3.9% fat
77 and 3.1% protein, at an average days in milk of 155. The dry cow ration contained straw, corn silage, and
78 protein supplement, and is considered a low calcium, low phosphorous, low potassium, high magnesium
79 diet that does not contain anionic salts. Historically, the farm averages less than 5% incident risk of
80 clinical hypocalcemia. This farm was chosen due to proximity with the veterinary clinic that assisted with
81 sample handling and analysis, as well as technical capability to collect and record relevant samples and
82 data.
83 Cows were randomly allocated to receive either the Calboost bolus (CB) or Bovikalc bolus (BK) according
84 to a randomization sheet that was generated using the RAND command in Microsoft Excel. In this
85 experiment, a positive control was used, as the practice of oral calcium supplementation at calving is an
86 industry-standard recommendation, and few producers capable of conducting field-based research are
87 willing to accept the risk of not prophylactically treating all mature cows with oral calcium. First lactation
88 cows, and those animals whose calvings were unobserved were excluded from enrollment. The farm
89 staff was responsible for bolus administration, blood sample collection and storage, and reporting any
90 disease events. They were blinded to the treatment groups throughout the trial.
91 The bolus was given orally at calving and again at 12 hours post-calving. Blood samples were collected
92 via venipuncture of the coccygeal vein. Blood was taken at 0, 1, 6, 12, 13, and 24 hours relative to
93 calving. Due to practicalities associated with sampling enrolled cows, the sampling window was allowed
94 to vary by ± 1 hour of the protocol designation. Samples were allowed to clot, centrifuged for 5 minutes
95 at 800 g, and serum was transferred into sterile storage vials. The vials were stored at -20°C until

96 submitted for analysis. Samples were analyzed to determine total serum calcium via a photometric
97 method (Abaxis Vetscan vs2, Abaxis Inc., Union City, CA).
98 Following treatments, participating farm personnel were instructed to record treatment assignment and
99 all pertinent health and disease events (including death and culling) for all cows that had calved after
100 study commencement.

101 Data collection

102 Variables of interest for analysis included date and time of calving, calcium bolus administration, and
103 blood collection, lactation number, dystocia, non-lactating days prior to calving, disease dates,
104 culling/death dates, and occurrence of twin pregnancies.

105 Statistical analysis

106 All datasets were imported as comma-separated files into the STATA 14 statistical software program
107 (StataCorp LP, College Station, TX) for statistical analysis. The cow was considered the unit of analysis for
108 this study.

109 Descriptive statistics and univariable analysis

110 Descriptive statistics were generated for the final dataset. Serum calcium, phosphorous, and magnesium
111 levels were explored through graphical plots. Specific comparisons between the serum calcium, days
112 dry, lactation, season of sampling, and the presence of twins at calving in CB and BK cows were made,
113 with univariable statistical comparisons made using Fisher exact tests or Kruskall-Wallis one-way
114 analysis of variance.
115 Statistical comparisons were made between potential predictor variables and outcomes of interest. For
116 this study, the outcome of interest was serum calcium level (in mmol/L). Univariable linear regression
117 modelling was employed to study variables of interest. Variables explored for each model included:
118 treatment group (CB vs BK), lactation group (2 and over 3), non-lactating days prior to parturition,

season of calving, sampling time (0, 1, 6, 12, 13, and 24 hours relative to calving), dystocia (0 = eutotic calving, 1 = dystotic calving), and presence of twins at calving. Variables that had moderate statistical associations with the outcome of interest (defined at a liberal P-value < 0.2) were included in subsequent multivariable models.

Multivariable regression analysis

A repeated measures mixed linear regression model was constructed, using random effects to model sample number within cow, using a first-order autoregressive correlation structure between time points. A step-wise backwards elimination process was employed, where variables identified as potentially associated with the outcome of interest in the initial univariable screening were included in a full multivariable model. Those variables having P-values for partial F-tests or type III tests of fixed effects greater than 0.05 were eliminated from the model after assessing whether they were part of biologically plausible interaction terms or had a confounding effect on the outcome of interest (e.g. a > 20% change in coefficient values when the term is removed from the model). Continuous variables were assessed for linearity with predicted model outcomes through visually inspecting a LOWESS curve (local weight scatterplot smoothing) for linear relationship, as well as the significance (P < 0.05) of a quadratic term in the model. If a continuous variable did not have a linear relationship with the outcome of interest, it was subsequently categorized based on biologically relevant cut-points, or if appropriate, a quadratic term was retained in the model. Variables retained in the final model were assessed for collinearity through the examination of Pearson or Spearman rank-order correlation coefficients. When high correlation was found between variables (> 0.6), the most biologically appropriate variable was chosen for inclusion in the final model. Standardized residuals were generated and visually assessed for normality and homoscedasticity for the linear mixed regression model. If the residuals were heteroskedastic or not normally distributed, appropriate transformations were performed on the outcome of interest.

Results and Discussion

Table 1 outlines the descriptive characteristics of trial animals. The BK group had a lower absolute number of animals in their second and third lactation, although this difference was not statistically significant. The twinning rate in each group was relatively low, with 2 twin calvings in the BK group and 1 in the CB group. Mean non-lactating days was numerically greater for CB cows, but this difference was not statistically significant.

Table 1. Descriptive demographic comparisons between CalBoost (CB) and Bovikalc (BK) of cows enrolled on a commercial dairy farm in southwestern Ontario, Canada.

Variable	CalBoost	BoviKalc	P-Value
Lactation (frequency)			
2nd and 3rd lactation	5	7	0.65
> 3rd lactation	5	3	
Calving Season (frequency)			
March-April	3	1	0.41
May-July	1	3	
August-September	6	6	
Twins (frequency)			
Yes	1	2	1.00
No	9	8	
Mean Days Dry (SE)	73.2 (8.1)	61.3 (6.3)	0.23

152 Figure 1. a. outlines a descriptive comparison in serum calcium levels between CB and BK-treated
153 animals. Of note, CB cows had lower serum calcium at parturition relative to BK animals, but the CB
154 group had numerically higher serum calcium levels in subsequent sampling times (Figure 1.a.). Figures
155 1.b. and 1.c. outline raw mean (SD) serum phosphorus and magnesium levels measured in study cows,
156 respectively. There were no significant differences between CB and BK groups for all blood metabolites
157 ($P > 0.05$) measured on univariable analysis.
158
159 **Figure 1. a. Raw mean (SD) serum calcium (mmol/L) plot of Calboost (solid line) and Bovikalc (dashed**
160 **line) groups over the first 24 hours following calving.**
161
162 **Figure 1. b. Raw mean (SD) serum phosphorus (mmol/L) plot of Calboost (solid line) and Bovikalc**
163 **(dashed line) groups over the first 24 hours following calving.**
164
165 **Figure 1. c. Raw mean (SD) serum magnesium (mmol/L) plot of Calboost (solid line) and Bovikalc**
166 **(dashed line) groups over the first 24 hours following calving.**
167
168 Of note, there were 2 animals of 20 that were sampled outside of the appropriate window (time ± 1
169 hour) for the 12-hour samples, both of which were in the CB treatment group. Analyses were conducted
170 both including and excluding these animals, with no bearing on the outcome of the experiment.
171 Therefore, the data for these animals remained in the analysis (N = 20) to avoid excessive model
172 overfitting.
173 Residual analysis from raw serum calcium data showed considerable heteroskedasticity, therefore
174 natural logarithms were calculated for each calcium value. Upon subsequent iterations of the model,

175 residuals were homoscedastic and normally distributed with this transformation. Multivariable,

176 repeated measures linear regression model output is given in Table 2.

177 **Table 2. Multivariable, repeated measures linear regression model output evaluating the effect that**

178 **Calboost (CB) relative to Bovikalc (BK) has on log-transformed serum calcium values within the first 24**

179 **hours following parturition.**

		Coefficient	SE	P-Value	95% Confidence Interval	
Treatment						
	BK[a]	Referent				
	CB[b]	0.03	0.05	0.50	-0.06	0.12
Sample No.						
	1	Referent				
	2	0.18	0.05	0.001	0.08	0.29
	3	0.07	0.06	0.23	-0.04	0.18
	4	0.04	0.06	0.52	-0.08	0.15
	5	0.01	0.06	0.80	-0.10	0.13
	6	-0.01	0.06	0.82	-0.13	0.10
Calving Month						
	March-April	Referent				
	May-July	-0.04	0.07	0.54	-0.17	0.09
	August-September	0.16	0.05	0.002	0.06	0.27
Days Dry		0.01	0.00	<0.001	0.01	0.02

Days Dry (quadratic)	0.00007	0.00002	0.001	0.00	0.00
Cow Lactation:					
2nd and 3rd	Referent				
3rd+	-0.08	0.05	0.11	-0.17	0.02
Constant	-0.07	0.15	0.65	-0.36	0.23

[a]BK = Bovikalc; [b]CB = Calboost

Serum calcium levels did not differ at any point between BK- and CB-treated cows (P = 0.50). Serum calcium levels rose dramatically at the 1-hour mark following parturition; however, these levels subsequently dropped and were not significantly different from baseline (t = 0) levels for the remainder of the 24-hour sampling period. Cows that calved in the months of August and September had significantly higher serum calcium levels (P = 0.002) relative to cows calving in March and April. Older cows tended (P = 0.11) to have lower serum calcium levels. This variable was included in the model, as it had a significant confounding effect (> 20% change in coefficient value) on the treatment variable of interest (CB versus BK-treatment). Previous research has noted older cows to be at an increased risk of clinical milk fever [1]. Overall, Figure 2 outlines serum calcium levels for BK- and CB-treated cows over the experimental period.

Figure 2. Least square mean (SE) serum calcium values for Calboost (solid line) and Bovikalc (dashed line) treated cows over the first 24 hours following parturition.

196 The manufacturing and marketing of novel supplements (i.e. mineral boluses, vitamins, botanicals) are
197 not subjected to as rigorous a regulatory process relative to veterinary pharmaceutical products. As
198 such, there is a paucity of peer-reviewed literature evaluating such novel products in the field. The
199 present study, though not designed to be a noninferiority or superiority study, does display that the
200 novel product is able to cause an elevation in serum calcium of recently calved cows of a magnitude
201 similar to another commercially available, efficacious product of similar composition.
202 Subclinical hypocalcemia (serum calcium levels < 2.0 mmol/l) is a pervasive issue in the modern dairy
203 industry. All cows in the current study experienced subclinical hypocalcemia. Previous research has
204 found that the incidence to be approximately 50% in mature (2nd or greater lactation) Holstein cows
205 [4,12]. Indeed, the risk of displaced abomasum, ketosis, retained placenta, and metritis was 3.7, 5.5,
206 3.4, and 4.3 times more likely, respectively, in cows that experience subclinical hypocalcemia relative to
207 normocalcemic cohorts [13]. Overall, the costs associated with subclinical hypocalcemia are estimated
208 to be approximately $125 per case [2]. For the average 500 cow herd, assuming 65% of the herd is in
209 their 2nd lactation or greater, and a 50% incidence of subclinical mastitis, the cost would be
210 approximately $20,312.50. Clearly, significant efforts should be made to prevent such losses.
211 Cows that received both calcium supplements experienced significant increases in serum calcium levels;
212 however, these levels were not sustained for the remainder of the 24 hours following parturition. These
213 results are consistent with recent literature that found oral calcium supplementation effectively
214 increased serum calcium levels at the 1 and 24 hours postpartum relative to untreated controls [8,11].
215 One important difference between the current experiment and the cited studies was the use of negative
216 control animals. As the current study was a pilot field-based trial, and calcium supplementation is a
217 pervasive practice throughout the industry, it was difficult (potentially unethical) to recruit a suitable
218 herd that would allow cows at high risk for hypocalcemia to remain untreated. On the other hand,
219 numerous studies evaluating postpartum, serial serum calcium levels in cows not receiving

220 supplemental calcium either remain constant or significantly decrease relative to baseline levels at
221 calving [8,11,14–16]. Therefore, although no definitive conclusions can be made, it would be a fair
222 assumption that had a negative control group been included in the experiment, their serum calcium
223 would likely not have increased at the 1-hour sample post-calving, and that calcium-supplemented cows
224 would likely have had higher serum calcium levels at this timepoint.

225 Conclusion

226 Subclinical hypocalcemia is a prevalent disorder of the modern commercial dairy cow. The current pilot
227 study found oral supplementation with two commercially available calcium boluses was associated with
228 a significant increase in serum calcium levels at 1-hour post-calving.

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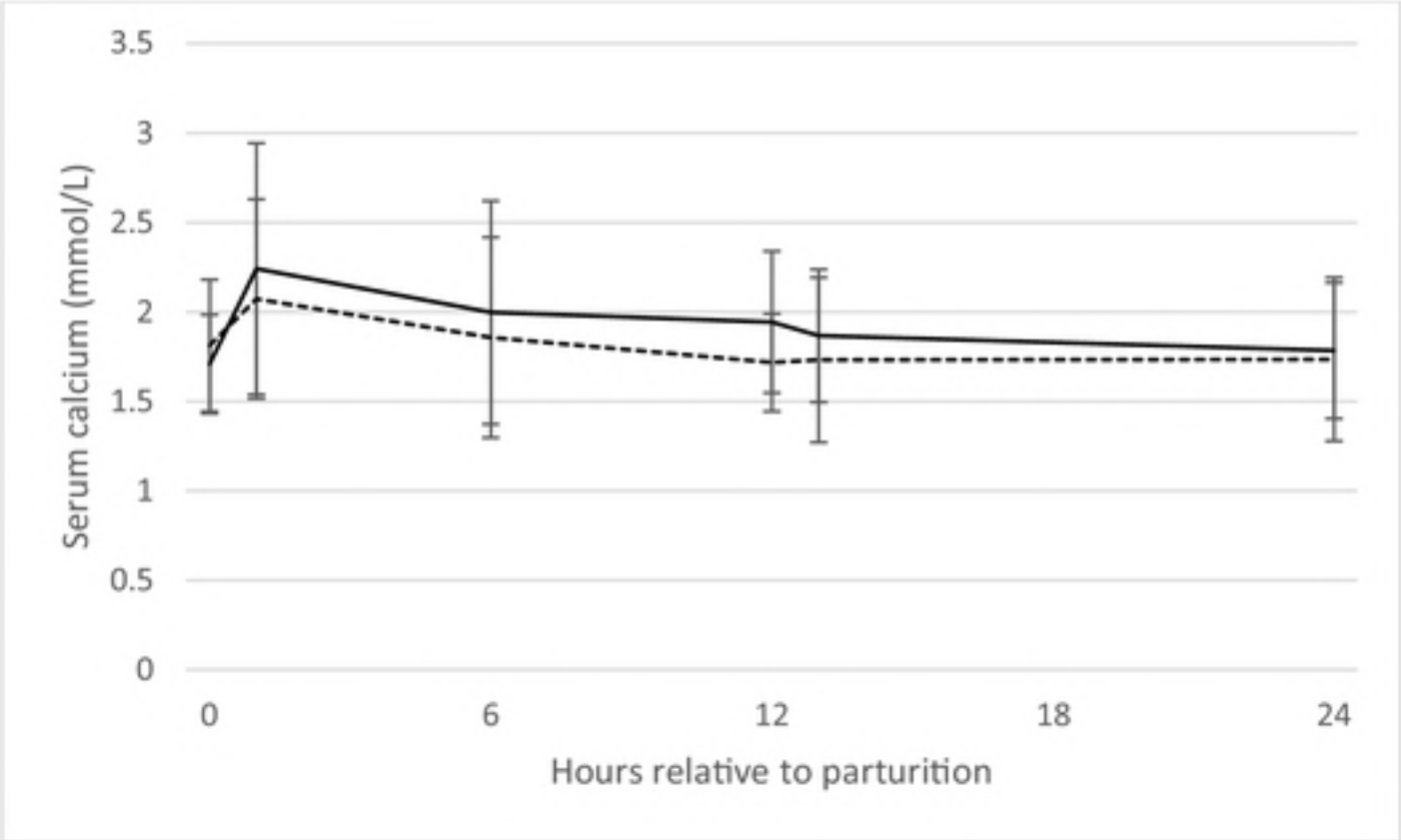
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3
2.5
2
1.5
1
0.5
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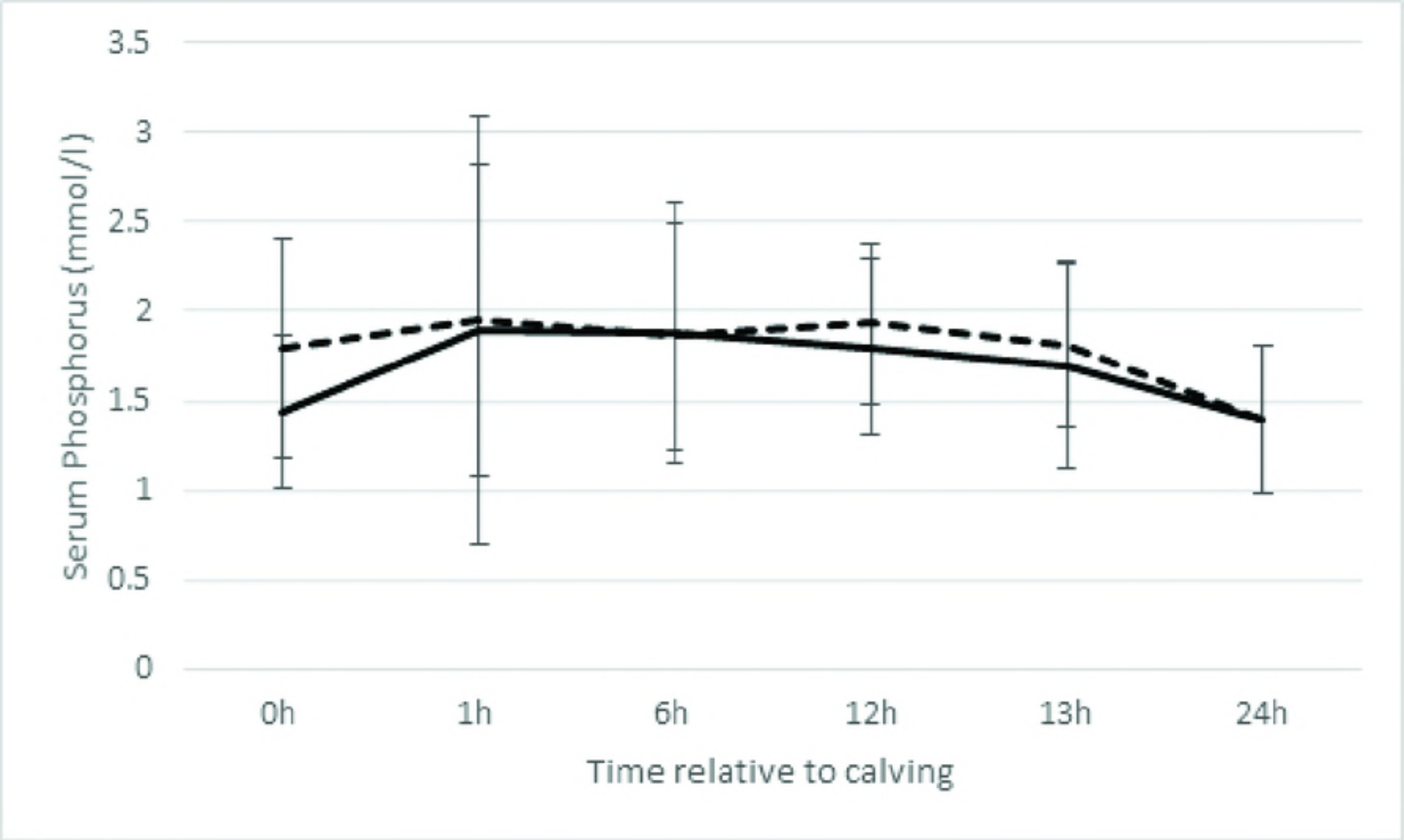
Serum calcium (mmol/L)
0
6
12
18
24
Hours relative to parturition

Figure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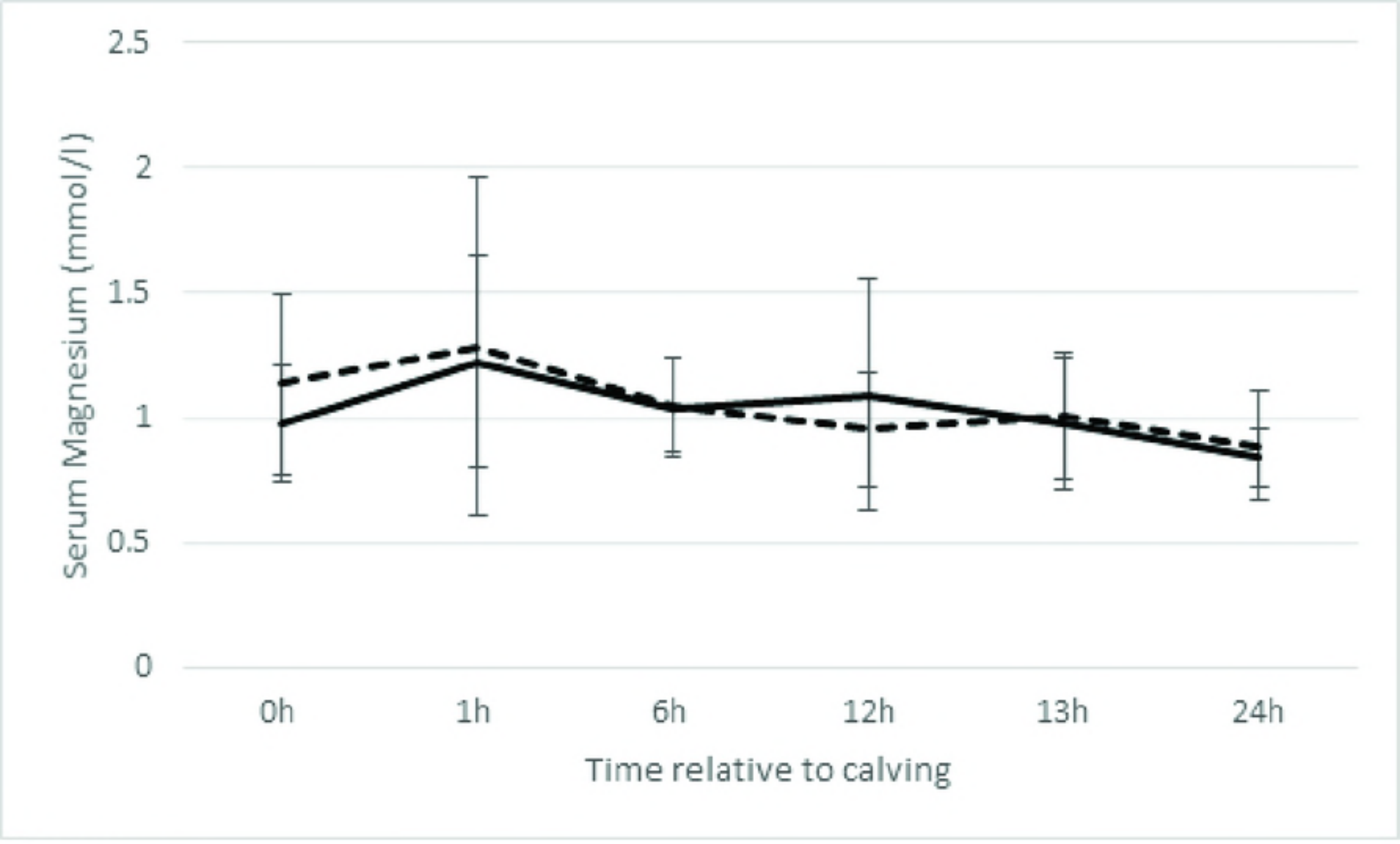
Serum Phosphorus (mmol/l)
0h
1h
6h
12h
13h
24h
Time relative to calving

Figure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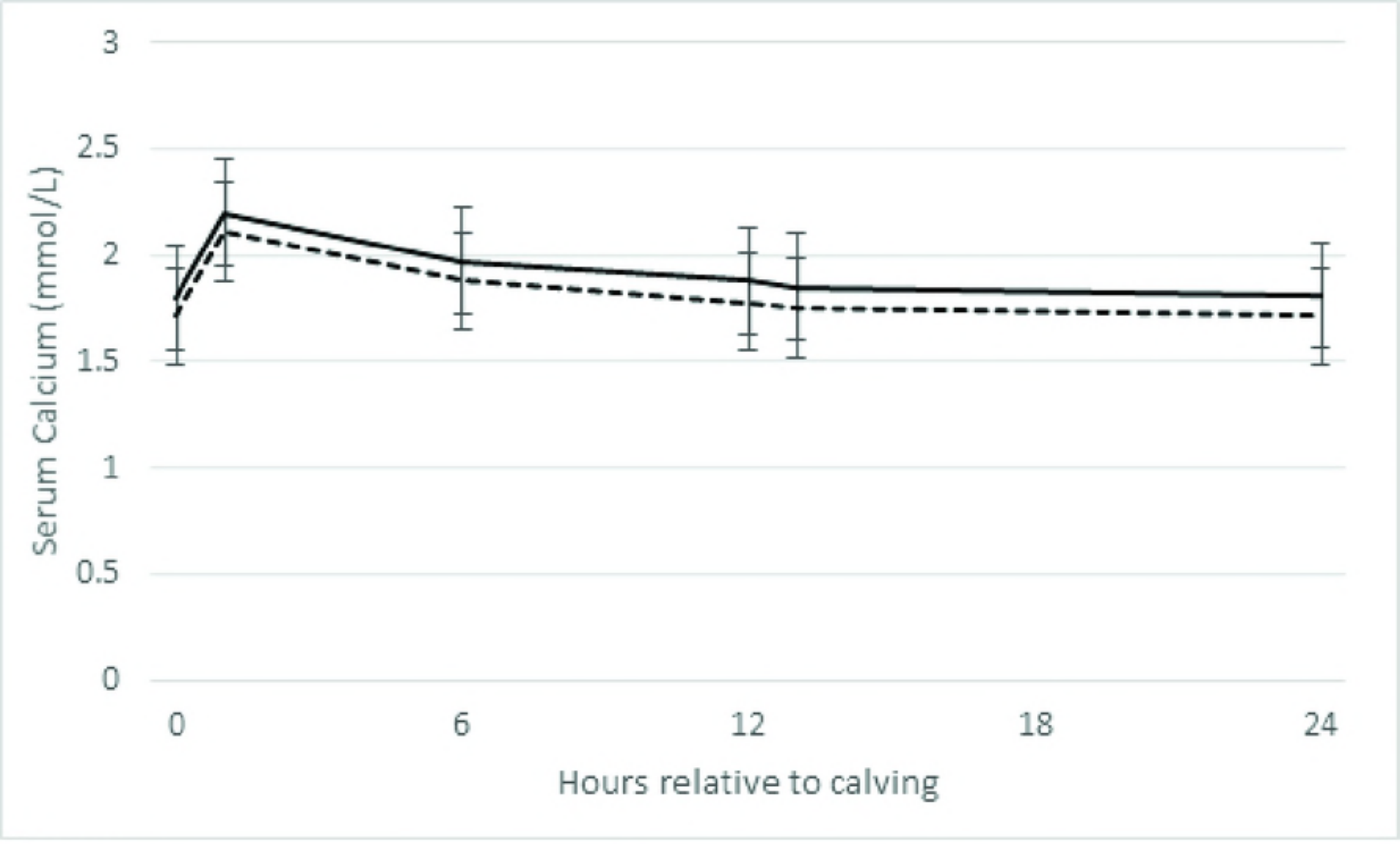
Serum Magnesium (mmol/l)
0h
1h
6h
12h
13h
24h
Time relative to calving

Figure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Serum Calcium (mmol/L)
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Hours relative to calving

Figure